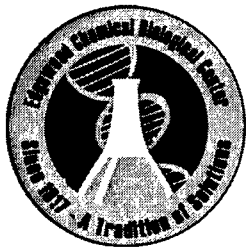

EDGEWOOD

CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND

ECBC-TR-460

TOXICITY SCREENING OF HYDROLYZED H, HD, AND HT USING THE BIOLUMINESCENT MARINE BACTERIUM, *VIBRIO FISCHERI,* BY MEANS OF MICROTOX ASSAY

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January 2006

ABERDEEN PROVING GROUND, MD 21010-5424

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-01-2006	Final	Jan 1996 - Mar 2005

4. TITLE AND SUBTITLE
Toxicity Screening of Hydrolyzed H, HD, and HT Using the Bioluminescent Marine Bacterium, *Vibrio Fischeri,* by Means of Microtoxic Assay

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Haley, Mark V.; and Checkai, Ronald T.

5d. PROJECT NUMBER
5VEWMH

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
DIR, ECBC, ATTN: AMSRD-ECB-RT-TE/AMSRD-ECB-RT-TV, APG, MD 21010-5424

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-460

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The U.S. Army Edgewood Chemical Biological Center developed an alternative method for disposal of the chemical agent, sulfur mustard. The mineralization of HD through hot water hydrolysis with subsequent neutralization using NaOH, followed by biodegradation, has been demonstrated to be an effective technology at the Aberdeen Chemical Disposal Facility (ABCDF). In Assembled Chemical Weapons Alternative sponsored testing, the mineralization process (reaction with hot water followed by neutralization using NaOH) has been applied to three grades of the vesicant chemical agent sulfur mustard, H, HD, and HT, at various feed loading concentrations. These three grades were obtained from projectiles, not from ton containers as was the case in application of the ABCDF technology. This research compared the toxicity of hydrolyzed neutralized mustard agent grades H, HD, and HT, using the marine bacterium *Vibrio fischeri* in Microtox bioassays (MTX). The 3.8%HT-Hydrolysate, 3.8% HD-Hydrolysate, and 1% H-Hydrolysate all had similar EC50 toxicity values on the basis of 5-min MTX bioassay results and were approximately five times more toxic than the 1.3% HD-Hydrolysate. The 8.6% H-Hydrolysate and the 8.6% HD-Hydrolysate were the most toxic of the samples tested, and were approximately 32 and 14 times more toxic, respectively, than the 1.3% HD-Hydrolysate.

15. SUBJECT TERMS

H-Hydrolysate	HD-Hydrolysate	EC50
HT-Hydrolysate	Microtox	Toxicity

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Sandra J. Johnson
U	U	U	UL	13	19b. TELEPHONE NUMBER *(include area code)* (410) 436-2914

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PREFACE

The work described in this report was authorized under Project No. 5VEWMH, Assembled Chemical Weapons Assessment Program. This work was started in January 1996 and completed in March 2005.

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TOXICITY SCREENING OF HYDROLYZED H, HD, AND HT USING THE BIOLUMINESCENT MARINE BACTERIUM, *VIBRIO FISCHERI,* BY MEANS OF MICROTOX ASSAY

1. INTRODUCTION

The purpose of these investigations was to compare the toxicity of various hydrolyzed neutralized grades of the chemical agent material sulfur mustard (H, HD, and HT) using the Microtox Assay (MTX). Some of the data listed in this paper were generated during the Alternative Technology (Alt. Tech.) Program that used hydrolyzed HD (obtained from ton containers at Aberdeen Proving Ground, MD) as bioreactor feed material.[1,2] The majority of the data presented in this report were generated under the current Assembled Chemical Weapons Alternative (ACWA) program. The mustard grades in these ACWA studies were: 1) H, Levinstein mustard, obtained from 155 mm H projectiles; 2) HD, obtained from 4.2" HD mortar projectiles; and 3) HT, obtained from 4.2" HT mortar projectiles. All projectiles were from lots stored at Desert Chemical Depot (DCD).

The investigators selected the bioluminescent marine bacterium, *Vibrio fischeri*, by means of the MTX, to screen for toxicity. The assay is inexpensive to run, the bacteria are sensitive to hydrolysis byproducts, small sample volumes are required for testing, and the marine bacteria *Vibrio fischeri* tolerate high salt concentrations.

Hydrolysates of the following chemical agents were used in testing: H [Bis-(2-Chloroethyl)sulfide; Levinstein mustard], HD [Bis-(2-Chloroethyl) sulfide; distilled mustard], and HT [60% Bis-(2-Chloroethyl)sulfide: 20% bis[2-(2-chloroethylthio)ethyl] ether; 60:20 HD:T]. According to Army record, HT is composed of 60 wt% HD and 40 wt% T, however the HT obtained and used in this study had an HD:T chemical agent ratio of 60:20 (wt:wt) respectively. The constituents of the remainder (20 wt%) are impurities and degradation products. It was also postulated that the HD:T ratio was close to 60:20 wt% in the U.S. stockpiles of HT projectiles when the munitions were first filled.

Most of the samples of hydrolyzed neutralized grades of sulfur mustard were dark in color and contained a high concentration of suspended particulate. These samples were allowed to settle, and only the clear aqueous fraction was used in toxicity testing.

2. MICROTOX ASSAY TEST PROCEDURES

The MTX assay[3] exposes bioluminescent marine bacteria (*Vibrio fischeri*, NRRL B-11177), to a sample of unknown toxicity, so that changes in the output of bioluminescent light by the bacteria may be measured as the means of determining the level of toxic effects on the bacterial organisms. Under proper test conditions, the reduction in light output is a direct indication of metabolic inhibition. The bacteria were cultured by Azur Environmental[3] and shipped in lyophilized form. The bacteria (stored frozen) were re-hydrated immediately before testing. Individual assays were performed in a temperature-controlled photometer using glass cuvettes containing 1 mL of sample. For optimum accuracy in predicting toxicity, the bioassay must have a minimum of four dilutions exhibiting a dose response. At 5 and 15 min, the control and treatment groups were measured for light output. Data were analyzed using the MTX 100% test protocol software to determine the EC_{50}, the effective concentration causing a 50% reduction in light output.

Many of the samples were dark and contained a high concentration of suspended solids. Suspended solids can interfere with the detection of light that is produced by the bacteria and yield skewed unrepresentative results. Therefore suspended solids were removed from samples by allowing solids to settle before sampling the clear aqueous fraction for toxicity testing. Sample parameters (pH and salinity) were measured and adjusted as needed. The pH was adjusted using 10% HCl, and salinity adjustments were made by adding sodium chloride directly to the sample.

Quality Assurance/Quality Control (QA/QC) testing was conducted using phenol as a standard toxicant. Using a standard whose toxicity is well known confirms the health of the test organism, and also checks the performance of the entire MTX system. The acceptable toxicity range for the EC_{50} value using the culture-lot of *Vibrio fischeri* used in these investigations was between 13 and 26 ppm (mg/L) for the phenol standard, as prescribed by Azur Environmental.[3] If the test result for the EC_{50} value of the phenol standard was outside this range, a new phenol standard was prepared and tested. If the standard result was still out of the range, a new batch of bacteria from the same culture-lot was prepared, and the phenol standard re-tested.

3. RESULTS

The MTX bioassay results, and sample identification numbers, are listed in Table 1. Using the phenol standard and the culture-lot of *Vibrio fischeri* used in these investigations, all QA/QC results fell within the acceptable range for phenol EC_{50} values (between 13 and 26 ppm) for 5-min exposure MTX bioassays.

On average, the order from least toxic to most toxic, on the basis of 5-min MTX bioassay results, is as follows: 1.3% HD- Hydrolysate <3.8% HT-Hydrolysate $\leq$1% H-Hydrolysate $\leq$3.8% HD-Hydrolysate <8.6% HD-Hydrolysate <8.6% H-Hydrolysate (Table 2). The 3.8% HT-Hydrolysate, 3.8% HD-Hydrolysate, and 1% H-Hydrolysate had similar EC_{50} toxicity values and were approximately five times more toxic than the 1.3% HD-Hydrolysate. The 8.6% H-Hydrolysate and the 8.6 % HD-Hydrolysate were the most toxic of the samples tested, and were 32 and 14 times more toxic than the 1.3% HD-Hydrolysate. For comparison, Table 2 also lists the respective toxicities of thiodiglycol, acetone, and methanol measured as 5-min EC_{50} values. The respective 3.8% HD-Hydrolysate, 3.8% HT-Hydrolysate, and 1% H-Hydrolysate are all similar in degree of toxicity to that of thiodiglycol. The 1.3% HD-Hydrolysate is similar in degree of toxicity to that of acetone, as 5-min EC_{50} values.

4. DISCUSSION

The respective toxicity results of the reference materials were ranked using the Chemical Scoring System for Hazard and Exposure Identification.[4] This system is typically used in the preliminary screening process, and is not intended to be a substitute for more complete risk assessment. The system assigns a score based on the acute toxicity data, using mg/L units. Using the density of thiodiglycol, acetone, and methanol, respectively, the %vol/vol units can be converted to milligram/liter for use in the scoring system.

The scoring system developed by O'Bryan and Ross[4] does not rank the scores using common terms typically used in mammalian toxicity rankings. The U.S. Fish and Wildlife Service (USFWS) published a Research Information Bulletin[6] suggesting relative aquatic toxicity terms based on EC_{50} data. The ranking system considers EC_{50} results greater than 1000 ppm (mg/L) to be "Relatively Harmless" and results less than 0.01 ppm (mg/L) as "Super Toxic." Similar descriptive rankings are used by Kamrin.[6] In Table 3 the respective toxicities for phenol, thiodiglycol, acetone, and methanol have been scored and ranked based on the EC_{50} results from the MTX bioassays.

Thiodiglycol is similar in toxicity to 3.8% HT Hydrolysate, 1% H Hydrolysate, and 3.8% HD Hydrolysate. Using the Chemical Scoring System for Hazard and Exposure Identification, thiodiglycol is considered to be relatively harmless to the MTX organism *Vibrio fischei*. On the basis of the scoring and ranking of thiodiglycol and drawing upon direct comparisons to the 3.8% HT-Hydrolysate, 1% H-Hydrolysate, and 3.8% HD-Hydrolysate, all are designated relatively harmless to the marine bacterium *Vibrio fischeri*. Acetone is similar in toxicity to 1.3% HD-Hydrolysate. Using the scoring and ranking of acetone in a direct comparison to the toxicity results for 1.3% HD- Hydrolysate, both may also be considered to be relatively harmless. The 8.6% H-Hydrolysate and the 8.6% HD-Hydrolysate were the two most toxic samples tested and were approximately 32 and 14 times more toxic, respectively, than 1.3% HD-Hydrolysate.

Table 1. Compiled Data from Microtox Bioassay Toxicity Testing of Hydrolyzed HD, HT, and H for 5- and 15-Min Exposures, EC_{50} (% vol/vol)

Date	Sample ID	Sample	5-Min EC_{50} (95%C.I.)	15-Min EC_{50} (95%C.I.)
1-16-96	Harvey60 OTH22295[7]	1.3% HD-Hydrolysate[a]	3.9% (3.3-4.6)	4.6% (3.9-5.3)
2-21-96	P-4A-07-HW-0456D	1.3% HD-Hydrolysate[a]	2.2% (0.1-4.2)	2.1% (0.1-47)
	P-4B-07-HW-0456D	1.3% HD-Hydrolysate[a]	3.5% (1.6-7.8)	4.5% (1.8-11.1)
3-31-01	PBHY25HD01AX (ACWA)	3.8% HD-Hydrolysate	0.6% (0.5-0.8)	0.7% (0.6-1.0)
	PBHY25HD01AD	3.8% HD-Hydrolysate	0.7% (0.5-1.0)	1.0% (0.4-2.1)
	PBHY25HD1AAD	3.8% HD-Hydrolysate	0.9% (0.7-1.2)	1.2% (1.0-1.5)
	PBHY25HD02AX	3.8% HD-Hydrolysate	0.4% (0.3-0.4)	0.5% (0.4-0.6)
	PBHY25HD01BX	3.8% HD-Hydrolysate	0.3% (0.3-0.4)	0.4% (0.4-0.5)
2-5-03	PBHY25HX01AD (ACWA)	3.8% HT-Hydrolysate	0.6% (0.5-0.7)	0.9% (0.8-1.0)
	PBHY25HX01AX	3.8% HT-Hydrolysate	0.8% (0.7-1.0)	0.9% (0.8-1.1)
	PBHY25HX01BX	3.8% HT-Hydrolysate	0.9% (0.7-1.1)	1.0% (0.8-1.3)
	PBHY25HX01CD	3.8% HT-Hydrolysate	0.8% (0.7-1.1)	1.1% (1.0-1.3)
	PBHY25HX01CX	3.8% HT-Hydrolysate	0.7% (0.6-0.8)	0.9% (0.8-1.0)
	PBHY25HX02AD	3.8% HT-Hydrolysate	0.7% (0.6-0.8)	0.9% (0.8-1.0)
	PBHY25HX02AX	3.8% HT-Hydrolysate	0.6% (0.4-0.7)	0.8% (0.7-0.9)
8-5-03	PBMH25HL01AX (ACWA)	1% H-Hydrolysate[b]	0.6% (0.4-0.9)	0.6% (0.4-0.9)
	PBMH25HL01AD	1% H-Hydrolysate[b]	0.7% (0.5-0.8)	0.6% (0.5-0.8)
2-7-05	50426-87-05 (ACWA)[c]	8.6% H-Hydrolysate	0.1% (0.06-0.16)	0.1% (.04-0.14)
3-29-05	Q0605FLD040607-01[d]	8.6% HD-Hydrolysate	0.23% (0.17-0.29)	0.25% (0.18-0.34)

a. HD was obtained from ton containers stored in APG. The hydrolysis procedure was conducted at bench scale, in glass.
b. The ACWA testing failed to generate the planned 15 wt% H-hydrolysate. The 1 wt% H-loading was an estimate determined by researchers operating the bioreactor.
c The hydrolysate was generated at Battelle Laboratories using the H-feed supplied by ACWA. The H contained 70 wt% solid phase and 30 wt% liquid phase.
d. HD was obtained from ton containers stored in APG. The hydrolysis procedure was conducted at the ABCDF.

Table 2. Average 5-Min EC_{50} Values for the Various Hydrolysate Samples and Reference Materials

Sample	EC_{50} (%; vol/vol)	EC_{50} (mg/L)
1.3% HD-Hydrolysate	3.2%	-
3.8% HT-Hydrolysate	0.72%	-
1% H-Hydrolysate	0.65%	-
3.8% HD-Hydrolysate	0.58%	-
8.6% HD-Hydrolysate	0.23%	-
8.6% H-Hydrolysate	0.10%	-
Thiodiglycol [(2)]	0.45%	5,310
Acetone	2.3%	18,170
Methanol	5.2%	41,600

Table 3. Toxicity Scoring of Microtox Data Using the O'Bryan and Ross Chemical Scoring System for Hazard and Exposure Identification,[4] and Ranking Using USFWS System[5]

	5-Minute EC_{50} (mg/L)	Score (0-9; 9 being most toxic)	Ranking
Phenol	18.4 [7]	4-5	Slightly Toxic
Thiodiglycol	5,310	0	Relatively Harmless
Acetone	18,170	0	Relatively Harmless
Methano	41,600	0	Relatively Harmless

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